41. (New) A recombinant cell transduced with the vector of claim 33.

42. (New) A recombinant cell transduced with the vector of claim 34.

43. (New) A recombinant cell transduced with the vector of claim 35.

44. (New) A recombinant cell transduced with the vector of claim 36.

B2 Cont

45. (New) A recombinant cell transduced with the vector of claim 37.

46. (New) A recombinant cell transduced with the vector of claim 38.

REMARKS

Amendments

The specification is amended to correct a spelling error in "Myer" and to adopt the current National Library of Medicine journal citation nomenclature for *Computer Applications in the Biosciences* and to provide the current name of this journal. The new, revised claims recite the protease activity of the subject polypeptides described on p.3, lines 11-12; the definition of high stringency hybridization conditions described on p.8, lines 15-16 and p.20, lines 25-26; the expression vector / operably linked promoter language as described on p.7, lines 11-14. These amendments introduce no new matter.

The sequences (SEQ ID NOS:1-4) of the enclosed Sequence Listing are identical to the sequences (SEQ ID NOS:1-4, except that prior SEQ ID NO:2 is now SEQ ID NO:3 and prior SEQ ID NO:3 is now SEQ ID NO:2) disclosed in our priority application (60/023,491, filed Aug 7, 1996 and incorporated by reference in the subject application). The canceled Sequence Listing contained several typographical errors introduced when the original SEQ ID NOS were manually transcribed for input into PatentIn software.

In adherence with 37 CFR 1.821-1.825, this response is accompanied by a diskette containing SEQ ID NOS 01-04 in computer readable form and a paper copy of the sequence information. The computer readable Sequence Listing was prepared through the use of the software program "PatentIn" provided by the Patent and Trademark Office. The sequence

information recorded in computer readable form is identical to that of the written sequence listing submitted herewith. The sequence data of the enclosed Sequence Listing are all contained in the prior application 60/023,491, filed Aug 7, 1996. This submission introduces no new matter.

35USC112, first paragraph (enablement)

The issue re conservative modifications to SEQ ID NOS:3 (now SEQ ID NO:2) and 4 is respectfully traversed. The issue is whether the specification enables one of ordinary skill in the art to practice the invention as claimed without undue experimentation. In particular, while the Action notes that the specification provides guidance on making conservative amino acid substitutions, the Action suggests there is insufficient guidance on where conservative amino acid substitutions can be made in the recited SEQ ID NO:2 and 4 without affecting the function of the required enzymatic function of the protein. However, one does not need to know, a priori, the functional impact of any proposed conservative substitution, for the art provides convenient screening methods for confirming retention of the required protease activity. As explained in the specification, the required protease activity is readily detected in a number of well-established assay formats, e.g. Kato, *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, 89:9554-9558, cited at p.2, line 26; and Ashby and Rine (1995) *Methods in Enzymology* 230:235, cited at p.13, lines 2-4 (both enclosed). Ashby and Rine (supra at p.245), for example, describe a simple, direct chromatography assay of protease activity. Hence, for any given conservatively modified polypeptide, only a simple biochemical screen is required to confirm retention of the required protease activity. This routine screening for retained activity is well-within the bounds of experimentation permitted by 35USC112, and compares very favorably with that validated by the Federal Circuit in *In re Wands.*

The issue re conservative modifications to SEQ ID NOS:1 and 2 (now SEQ ID NO:3) is avoided by canceling this claimed subject matter.

35USC112, first paragraph (written description)

The issue re conservative substitutions is avoided by canceling this claimed subject matter. The present claims all provide structural and functional limitations for the claimed species. As presently claimed, the claimed subject matter is described in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application

was filed, had possession of the claimed invention. In its broadest recitation, the claimed invention is an expression vector comprising a promoter operably linked to an expressed polynucleotide which hybridizes under recited highly stringent conditions (hybridization and wash conditions selected to be 5° C lower than the thermal melting point (Tm) for said nucleic acid at a defined ionic strength and pH) to a nucleic acid which encodes a recited polypeptide which may be conservatively modified and has defined, recited function (mediates the proteolytic removal of an AAX tripeptide from a prenylated CAAX protein).

The claimed genus recites a compound limitation: the gene must hybridize with a nucleic acid which in turn must encode a recited polypeptide. Because of the degeneracy of the genetic code, there is a virtually unlimited number of nucleic acids which can encode a long polypeptide. While only one exemplary coding sequence is explicitly recited for each polypeptide sequence, the genetic code equally conveys to those of ordinary skill in the art, that one who conveys possession of a recited polypeptide sequence (at least since the elucidation of the genetic code), also inherently conveys possession of the genus of polynucleotides encoding such polypeptide. Generating other members of the genus involves no more than arbitrarily selecting alternative codons at one or more arbitrary positions. It would impossibly burden the public and to no benefit, if an applicant were to recite endless possible coding sequences.

Similarly with a long nucleic acid, there is a virtually unlimited number of genes which will hybridize under stringent conditions. Generating other members of the genus involves no more than arbitrarily selecting alternative nucleosides at one or more arbitrary positions, and if a substantial number of positions are changed, confirming that the novel gene does in fact still hybridize with the recited nucleic acid under the recited stringency. It would again impossibly burden the public and to no benefit, if an applicant were to recite endless possible stringently hybridizing sequences.

By reciting a polypeptide sequence in view of the genetic code, applicant was clearly in possession of the genus of polynucleotides which encode such sequence. Similarly, by reciting a nucleic acid sequence and hybridization conditions, applicant was clearly in possession of the genus of polynucleotides which hybridize with such nucleic acid under such conditions. Contemplating and producing other members of the genus involves as little as altering a nucleoside. Contrast this scenario to that of *UC v. Lilly*, where the practitioner was instructed to go out and clone de novo novel sequences from alternative species. In Lilly, the applicant

claimed a genus of every "vertebrate cDNA encoding insulin", while only disclosing the corresponding rat cDNA. Isolating other members of the claimed cDNA genus would involve de novo cloning from each species, as the claim encompasses sequences which are, or are the same as, sequences isolated from a given vertebrate species. The Federal Circuit determined that the rat cDNA did not reasonably convey possession of the genus of every vertebrate cDNA. In our facts, there is no claim to cDNA, nor any requirement that the practitioner isolate anything from nature. Instead, our scenario compares quite favorably with that of antibody claims, such as in *In re Wands*, wherein the practitioner is instructed to make and screen antibodies for binding affinity.

The issue re a deposit under the Budapest Treaty is avoided by canceling this claimed subject matter.

The issue re SEQ ID NO:1 not encoding SEQ ID NO:3 (now SEQ ID NO:2) has been avoided with the corrected Sequence Listing.

The issue re new matter is avoided by canceling this claimed subject matter.

35USC103(a)

The art rejections all rely on Rose, M. et al. (GenBank Database, Accession No. Z49617), which is dated Aug 11, 1997, more than a year after our Aug 7, 1996 priority date, and is hence not prior art. The examiner has hand-written on the NCBI printout "Public Availability: 10/6/95". Upon telephone inquiry, the Examiner indicated that the only support for his hand-written comment was a "creation-date" annotation associated with the GenBank entry. However, the same annotation continues that the entry was updated on Aug 11, 1997. The Examiner indicated that the Action is premised on an assumption that the relied upon sequence was published on Oct 6, 1995. Such an assumption goes farther than relying on manuscript submission dates instead of publication dates - a practice disclaimed by the Office - for with the latter, there is no assurance that the information was modified at all. Here, the evidence dictates that the entry purportedly created on Aug 11, 1997 is not the same as that created on Oct 6, 1995. The Action offers no evidence that the relied upon sequence was presented to GenBank or created or published at any time prior to Aug 11, 1997.

In any event, the entire yeast genome had been largely sequenced prior to the filing of our patent application, including the identification of thousands of ORFs which were not even known

to encode functional mRNA. Even if these ORFs contained an identical or substantially identical sequence, the claimed compositions would be neither anticipated nor obvious. First, even if a yeast chromosome sequence is determined (and it appears that a sequence encoding Afc1p (SEQ ID NO:2) is found on the yeast X chromosome), our claims do not encompass any chromosome. Second, our claims require that the coding sequence be operatively joined to a promoter. In the absence of any evidence for function, there would be no motivation to select out one of the thousands of yeast ORFs of unknown function, isolate what may or may not be a coding sequence, and operatively join it to a promoter.

Absent a prior art suggestion that SEQ ID NO:1 or 2 (now SEQ ID NO:3) encodes a protein of determined function sufficient to motivate the isolation, cloning and expression of such SEQ ID NO using the techniques of the cited Nozaki et al. (US Pat No 4,997,767) and Sambrook, J. et al. (Mol. Cloning, Cold Spring Harbor Press, p. 16.3-16.16), the claims are in compliance with 35USC102 and 103.

It has come to our attention that two US patents have recently issued describing human variants of the disclosed RCE1 and AFC1 (US Pat Nos.6,110,717 and 6,060,277, respectively). Both Patents have an earliest claimed priority date of June 24, 1997, nearly a year after ours.

The Examiner is invited to call the undersigned if he would like to amend the claims to clarify the foregoing or seeks further clarification of the claim language.

Applicants hereby petition for any necessary extension of time pursuant to 37 CFR 1.136(a). The Commissioner is hereby authorized to charge any fees or credit any overcharges relating to this communication to our Deposit Account No. 19-0750 (order no. B96-021-3).

Respectfully submitted,
SCIENCE & TECHNOLOGY LAW GROUP

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enc. Kato, *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, 89:9554-9558.
Ashby and Rine (1995) *Methods in Enzymology* 230:235.
Sequence Listing, 9 p.
CRF containing Sequence Listing (1 diskette)

[20] Ras and a-Factor Converting Enzyme

By MATTHEW N. ASHBY and JASPER RINE

Introduction

Proteins containing a carboxyl-terminal CaaX motif undergo posttranslational processing involving prenylation of the cysteine residue, proteolytic removal of the terminal three amino acids, and finally methylesterification of the prenylated cysteine.[1-3] In contrast to the protein prenyltransferases and the methyltransferase, isolation of the respective gene(s) encoding the C-terminal protease remains elusive. Nevertheless, many attributes of CaaX-proteolytic processing of the yeast Ras2 protein and the a-factor pheromone have been reported. For instance, *in vitro* processing assays of subcellular fractions from yeast identified three protease activities that can remove the terminal three amino acids from prenylated substrates.[4,5] Two of the enzymes do not show dependence on the substrate being prenylated. One of the prenyl-independent proteases is the vacuolar degradative protease carboxypeptidase Y,[5] and the other appears to be a zinc metalloendopeptidase that cleaves on the carboxyl side of hydrophobic residues near the C terminus of some peptides.[6] Because farnesylation must precede proteolysis,[7] prenylation dependence is an attribute expected of the protease that processes prenylated substrates *in vivo*. The third protease is dependent on a prenylated substrate, is tightly associated with membranes, and catalyzes an endoproteolytic scission releasing the terminal three amino acids intact as a tripeptide.[4] We refer to the membrane-bound endoprotease as RACE for Ras and a-factor converting enzyme.

An analogous mammalian enzyme has been described from rat[4,8,9] and bovine[10,11] tissues that also localizes to membranes and catalyzes an endo-

[1] S. Clarke, *Annu. Rev. Biochem.* **61**, 355 (1992).
[2] W. R. Schafer and J. Rine, *Annu. Rev. Genet.* **26**, 209 (1992).
[3] A. D. Cox and C. J. Der, *Curr. Opin. Cell Biol.* **4**, 1008 (1992).
[4] M. N. Ashby, D. S. King, and J. Rine, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4613 (1992).
[5] C. A. Hrycyna and S. Clarke, *J. Biol. Chem.* **267**, 10457 (1992).
[6] C. A. Hrycyna and S. Clarke, *Biochemistry* **32**, 11293 (1993).
[7] W. R. Schafer, C. E. Trueblood, C.-C. Yang, M. P. Mayer, S. Rosenberg, C. D. Poulter, S.-H. Kim, and J. Rine, *Science* **249**, 1133 (1990).
[8] G.-F. Jang, K. Yokoyama, and M. H. Gelb, *Biochemistry* **32**, 9500 (1993).
[9] L. Liu, G.-F. Jang, C. C. Farnsworth, K. Yokoyama, J. A. Glomset, and M. H. Gelb, this volume [16].
[10] Y.-T. Ma and R. R. Rando, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6275 (1992).
[11] Y.-T. Ma, B. A. Gilbert, and R. R. Rando, this volume [19].

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proteolytic cleavage of prenylated substrates. The endoprotease requires a prenylated substrate. Either a geranyl, farnesyl, or geranylgeranyl lipid can fulfill the prenylated substrate requirement of the mammalian endoprotease.[8,10] Thus, the presence of an isoprenoid group, but not the exact nature of the lipid, is critical for enzyme activity. The yeast and mammalian membrane endoproteases appear to share many properties including a highly conserved substrate specificity.[12] To date, all published *in vitro* data support the notion that the membrane-localized endoprotease is the physiologically relevant protease for processing C termini of prenylated substrates. However, the assignment must be considered tentative until a definitive *in vivo* involvement of the protease with prenylated substrates is established. We present a number of methods utilized successfully in this laboratory to isolate RACE-enriched yeast membrane preparations and describe quantitative and qualitative assays to determine CaaX-endoproteolytic processing *in vitro*.

Determination of Ras and a-Factor Converting Enzyme *in Vitro*

Source of Enzyme

As shown in Table I, yeast membrane preparations present a rich source of RACE. Below we describe a simple method for obtaining yeast membrane preparations that are 5- to 7-fold enriched for RACE activity over the level found in 2500 *g* supernatants of crude extracts. For reference we include a distribution of RACE activity in yeast subcellular fractions (see Table II) obtained by differential centrifugation (Fig. 1). The following method requires about 4–6 hr and has been employed successfully with numerous yeast strains.

Materials

YPD medium: 1% Bacto yeast extract, 2% Bacto-peptone, 2% dextrose
SST buffer: 0.3 *M* sorbitol, 0.1 *M* NaCl, 10 m*M* Tris-HCl, pH 7.4, 5 m*M* EDTA, pH 8, 1 m*M* phenylmethanesulfonyl fluoride (PMSF). The PMSF is added immediately prior to use from a 200 m*M* stock solution in methanol
Sterile water
Glass beads: 250–300 μm, acid washed and sterilized
Tubes: 50-ml screw-capped polypropylene, 15-ml Corex, and 13.5-ml (16 × 76 mm) Beckman (Palo Alto, CA) polyallomer ultracentrifugation tubes

[12] M. N. Ashby, V. L. Boyartchuk, and J. Rine, in preparation (1995).

TABLE I
SOURCES OF CaaX ENDOPROTEASE

Source[a]	Specific activity (pmol min^{-1} mg^{-1})	Ref.
Saccharomyces cerevisiae[b]		
JRY2594	974	*c*
BJ2168	3381[d]	*e*
Xenopus oocyte	33	*f*
Rat liver	17	*e*
	8[d]	*g*
Bovine liver	251[d]	*h*

[a] Extracts consisted of membrane preparations.
[b] Genotypes are as follows: JRY2594, *MATα ade2-101 his3-200 lys2-801 met ura3-52*; BJ2168, *MATa pep4-3 prb1-1122 prc1-407 leu2 trp1 ura3-52*.
[c] M. N. Ashby, D. S. King, and J. Rine, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4613 (1992).
[d] Specific activity is given as V_{max} determined from Lineweaver–Burke or Eadie–Hofstee plots.
[e] M. N. Ashby and J. Rine, unpublished data (1992).
[f] M. N. Ashby, R. Kim, D. S. King, M. Wu, S.-H. Kim, and J. Rine, unpublished data (1992).
[g] G.-F. Jang, K. Yokoyama, and M. H. Gelb, *Biochemistry* **32**, 9500 (1993).
[h] Y.-T. Ma and R. R. Rando, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6275 (1992).

21-Gauge needle with a 12-ml syringe
2.0 *M* Na_2CO_3, pH 11.5
10 ml Dounce homogenizer with a Teflon pestle (Wheaton, IL)

Method. Grow a 1-liter yeast culture in YPD medium at 30° to an OD_{600} of 0.6–1 (1 OD unit = 10^7 cells/ml; mid-log phase). Harvest the cells by centrifugation at 5000 *g* (5500 rpm in a Sorvall GS-3 rotor) for 15 min at 4°. Wash the cells by resuspending the sample in 200 ml chilled sterile water. Collect the cells by centrifugation as in the previous step. The packed cell volume should be approximately 2 ml. Resuspend the cell pellet with 5 ml SST buffer and transfer the cells to a 50-ml polypropylene tube.

Add 5 ml of sterile glass beads and place on ice for 10 min to chill thoroughly. Vortex the tube at maximum setting for 1 min, and replace the tube on ice for 1 min to rechill since vortexing will heat the contents of the tube. Repeat the vortex–ice incubation regimen five times. Transfer the cell lysate to a chilled 15-ml Corex tube utilizing a 21-gauge needle on a 12-ml syringe. This technique prevents the transfer of glass beads to the Corex tube.

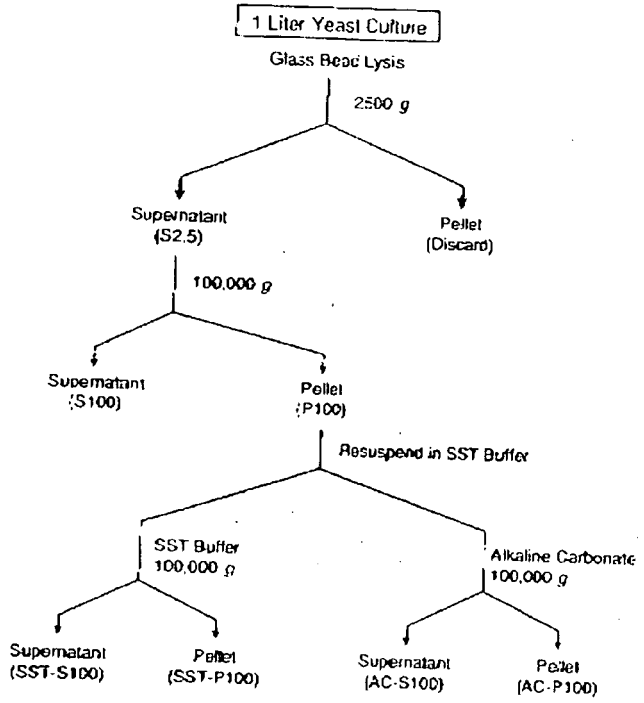

FIG. 1. Yeast extract fractionation scheme.

Centrifuge the lysate at 2500 *g* (4500 rpm in a Sorvall SS34 rotor) for 20 min at 4°. Transfer the 2500 *g* supernatant (S2.5) to another 15-ml Corex tube. Care must be taken not to transfer any of the pellet because the cellular debris would be carried through the procedure to the final fraction. Should any apparent spillover occur, the 2500 *g* centrifugation step must be repeated. Add 1/10 volume of 2 *M* Na_2CO_3, pH 11.5, and mix well. Transfer the S2.5 cell-free lysate to two 13.5-ml ultracentrifuge tubes.

Centrifuge the S2.5 lysate at 100,000 *g* (40,000 rpm in a 70.1 Ti rotor) for 1 hr at 2°. Discard the supernatant (S100) and gently wash the pellet (P100) two times with 10 ml cold SST buffer. Resuspend the pellet in 10 ml of cold SST buffer and transfer to a Dounce homogenizer on ice. The particulate matter should be uniformly homogenized by 5 strokes of a tight-fitting Teflon pestle. Centrifuge the sample again at 100,000 *g* for 1 hr at 2°. Repeat the same washing and resuspension procedure except resuspend the final membrane preparation in 2 ml cold SST buffer.

TABLE II
DISTRIBUTION OF RAS AND a-FACTOR CONVERTING ENZYME ACTIVITY[a]

Fraction	Total protein (mg)	Specific activity (pmol min^{-1} mg^{-1})	Total units[b]
S2.5	77	212	16,364
S100	32	32	1008
P100[c]			
SST-S100	2	16	35
SST-P100	41	296	12,136
AC-S100	21	16	336
AC-P100	10	1025	10,250

[a] Fractions were prepared by the scheme depicted in Fig. 1.
[b] One unit is defined as 1 pmol V[4,5-^{3}H]IA released from the peptide KWDPAC(*S-trans,trans*-farnesyl)V[4,5-^{3}H]IA per minute. Total units represents protease activity per 800-ml yeast culture grown in rich (YPD) medium to an OD_{600} of 1.
[c] The primary 100,000 *g* pellet was resuspended in SST buffer and divided into two tubes for the alkaline carbonate (AC) treatment. The values shown have been corrected for the division.

Finally, divide the alkaline carbonate-leached membrane preparation (AC-P100) into aliquots and freeze at −80°. Typically, the yield is between 2 and 4 mg protein per OD_{600} unit of cells. We have stored samples at −80° for over 1 year without significant loss of activity.

Considerations. Unlike the vacuolar proteases whose activities increase on entry of cells into stationary phase,[13] RACE activity decreases in stationary phase cells. Therefore, the cells should be harvested in mid-logarithmic phase to obtain high specific protease activity. For cell lysis we have utilized a French pressure cell and spheroplast–osmotic lysis[14] with similar yields as glass bead lysis. Efficiency of cell lysis can be monitored by visual inspection under a microscope at 200× magnification.

As shown in Table II, RACE fractionates as an integral membrane protein. Thus, alkaline carbonate (AC)-leaching of peripheral membrane proteins results in an approximately 3- to 4-fold increase in RACE specific activity in the AC-P100 fraction. We have found that sucrose gradient purification[15] of crude membranes (100,000 *g* pellet) yields equivalent RACE specific activity as AC-P100 preparations. Moreover, further alkaline carbonate treatment of the sucrose-purified membranes does not result in increased protease specific activity.

[13] E. W. Jones, this series, Vol. 194, p. 428.
[14] N. C. Walworth, B. Goud, H. Ruohola, and P. J. Novick, *Methods Cell Biol.* **31**, 535 (1989).
[15] R. Serrano, *FEBS Lett.* **156**, 11 (1983).

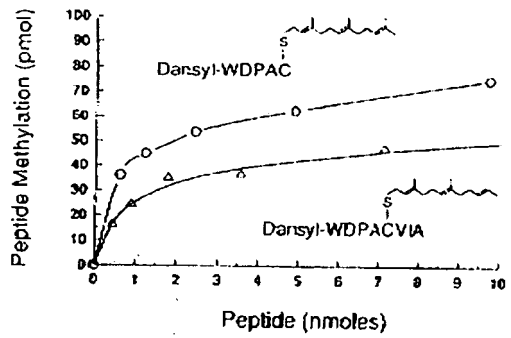

FIG. 2. Michaelis–Menten plot of methylation and coupled proteolysis–methylation of a farnesylated peptide. Varying amounts of a farnesylated a-factor peptide representing proteolyzed and unproteolyzed substrates were incubated with yeast crude membrane preparation (100,000 *g* pellet). Peptide carboxylmethylation was quantitated by alkaline hydrolysis of the methyl esters [R. C. Stephenson and S. Clarke, *J. Biol. Chem.* 267, 13314 (1992)].

Radiochemical Determination of Ras and a-Factor Converting Enzyme

Indirect Coupled Assay

Following prenylation and C-terminal proteolysis of CaaX sequences, the newly exposed α-carboxyl group of the prenylated cysteine is methylesterified by the *STE14*-encoded methyltransferase.[16] That enzyme requires a protein/peptide substrate that is prenylated and proteolytically processed to expose the carboxyl group of the farnesylated cysteine. We developed an indirect assay for RACE based on its ability to create, through proteolysis, a substrate for the methyltransferase. This coupled assay measures the amount of methyl groups transferred to the carboxyl terminus of a prenylated peptide. Methyltransferase activity is determined by quantitating the methyl groups as methanol released from the alkaline hydrolysis of the methyl ester product.[17] This method has great utility in that many peptides may be rapidly tested for protease and methyltransferase activity without having to synthesize radiolabeled peptides. A Michaelis–Menten plot of the methylation of peptides with and without the terminal tripeptide is shown in Fig. 2. The method described below is essentially the same as the *in vitro* methyltransferase assay described by Stephenson and Clarke.[18]

[16] C. A. Hrycyna, S. J. Wait, P. S. Backlund, Jr., and S. Michaelis, this volume [21].
[17] D. Chelsky, N. I. Gutterson, and D. E. Koshland, Jr., *Anal. Biochem.* 141, 143 (1984).
[18] R. C. Stephenson and S. Clarke, *J. Biol. Chem.* 265, 16248 (1990).

Materials

S-Adenosyl-L-[*methyl*-^{14}C]methionine (SAM) (47 mCi/mmol)
Peptides: Dansyl-WDPA(*S-trans,trans*-farnesyl)CVIA in 25% acetonitrile, Dansyl-WDPA(*S-trans,trans*-farnesyl)C in 25% acetonitrile
Reaction buffer: 100 m*M* Tris-HCl, pH 7.4, 1 m*M* PMSF, and 4 m*M* *o*-phenanthroline, prepared fresh
1 *M* NaOH/1% sodium dodecyl sulfate (SDS), made fresh
Filter paper (Bio-Rad, Richmond, CA; Cat. No. 165-0962) cut into 1.5 × 8 cm pleated strips

Method. Dispense 1 nmol of each peptide into 1.5-ml microcentrifuge tubes and evaporate to dryness under a light stream of nitrogen or in a Speed-Vac (Savant) concentrator. Resuspend peptides in 40 μl reaction buffer containing PMSF and *o*-phenanthroline. Dispense 1 nmol [^{14}C]SAM to each tube.

Initiate the reaction by adding 10 μl membrane preparation (50 μg protein). Incubate the reaction at 37° for 1 hr. Prepare the filter strips and scintillation vials while the reaction is in progress. Cut the filter paper into 1.5 × 8 cm strips and fold back and forth about 10 times. Place the pleated strip into the neck of a scintillation vial containing 5 ml aqueous scintillation fluid.

Terminate the reaction by adding 50 μl NaOH/SDS solution and mix well by pipetting up and down. Immediately transfer the entire reaction mix to the filter strip in the neck of the vial and cap the vial. After 2–4 hr at room temperature, carefully remove and discard the filter strips. Take care to keep the filter from contacting the scintillation fluid. The methyl esters formed in the reaction are hydrolyzed by the alkaline conditions, forming [^{14}C]methanol which diffuses into the scintillation fluid. Radioactivity is measured by liquid scintillation counting.

Considerations. A control assay without a farnesylated peptide substrate should be included in the experiment, and the value obtained from the reaction should be subtracted from the other reactions. The coupled assay is very specific, and control reactions incubated in the absence of farnesylated peptide acceptor typically produce less than 5% of the methylation measured in the presence of acceptor peptide substrate. Moreover, methyltransferase assays of membrane preparations from *ste14* mutant yeast strains indicates that the *STE14* methyltransferase accounts for more than 99% of the base-labile methyl esters detected in the assay.[19] However, an inherent weakness is that the coupled assay is indirect. Factors that affect methyltransferase activity will compromise proteolytic determinations. The methyltransferase is significantly more labile than is RACE, necessitating mea-

[19] C. A. Hrycyna, S. K. Sapperstein, S. Clarke, and S. Michaelis, *EMBO J.* 10, 1699 (1991).

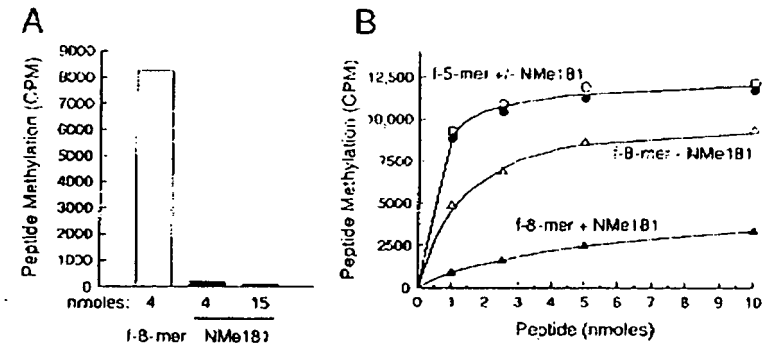

FIG. 3. Assessment of the RACE inhibitor NMe181 with the coupled proteolysis–methylation assay. (A) Proteolysis of the farnesylated 8-amino acid peptide Dansyl-WDPA (farnesyl)CVIA (f-8-mer) and the *N*-methyl peptide KGVFWDPA(farnesyl)C(*N*-methyl)VIA (NMe181) was determined by the indirect carboxylmethylation assay. (B) Processing of farnesylated peptides representing proteolyzed (f-5-mer, circles) and unproteolyzed (f-8-mer, triangles) substrates. Interaction of NMe181 with RACE and/or the methyltransferase was judged by its ability to inhibit methylation or coupled proteolysis–methylation of the f-5-mer or f-8-mer substrates, respectively. Peptide methylation was measured in the absence (open symbols) or presence (filled symbols) of 300 μM NMe181.

surement of methylation of farnesylated peptide lacking the terminal tripeptide as a standard. Additionally, the methyltransferase has about 90-fold less specific activity than RACE. Thus, relatively small changes in protease activity are not discernible from the coupled assay. Hrycyna and Clarke[5] have addressed this limitation in a similar assay by supplementing the coupled reaction with recombinant methyltransferase expressed in bacteria as an active TrpE–Ste14 fusion protein.[16]

As an example of an application of the coupled assay, we describe an experiment to determine whether a potential inhibitor, NMe181, is recognized and processed by RACE. NMe181 is a farnesylated a-factor peptide possessing an *N*-methyl modification at the amide linkage of the scissile peptide bond.[20] As shown in Fig. 3A, the presence of the *N*-methyl modification blocks proteolysis and, thus, methylation by 40- to 60-fold compared to an unmodified peptide. The defect is due to proteolysis because if cleavage had taken place the two peptides would have identical C termini and would therefore have been identical substrates for the methyltransfer-

[20] M. N. Ashby and J. Rine, in preparation (1995).
[21] R. J. Beynon and G. Salvesen, *in* "Proteolytic Enzymes: A Practical Approach" (R. Benyon and J. S. Bond, eds.), p. 241 (1989).

ase. In competition experiments (Fig. 3B) the presence of 300 μM NMe181 had no effect on the methylation of a farnesylated peptide lacking the terminal tripeptide, which indicated that NMe181 did not inhibit the methyltransferase. In contrast 300 μM NMe181 significantly inhibited methylation of a farnesylated peptide with the C-terminal tripeptide. Therefore, NMe181 specifically bound to RACE but did not undergo proteolytic cleavage of the tripeptide. These results have since been confirmed by more rigorous quantitative kinetic analyses with the direct RACE assay described below.

Direct Assay

Direct measurement of proteolysis alleviates the complexities associated with the coupled assay. The basis for the assay is that once a farnesylated peptide has been proteolytically cleaved, the C-terminal tripeptide can readily be separated from the prenylated product and, if radiolabeled, can be measured directly. The synthesis of prenylated peptides containing radiolabeled C-terminal residues has been described.[4,9,11] In this laboratory the assay has proved to be the most rapid and accurate method of determining RACE activity. Figure 4 illustrates the substrate concentration dependence of endoproteolytic cleavage of a farnesylated substrate by RACE in the direct assay.

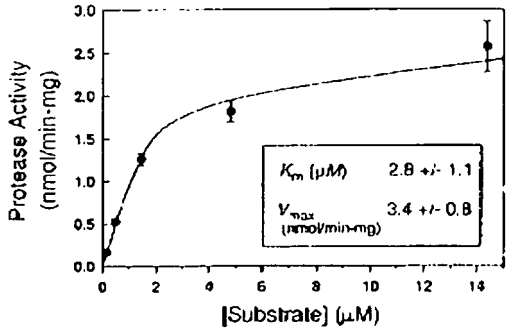

FIG. 4. Michaelis–Menten plot of endoproteolytic cleavage of an a-factor farnesylated peptide. One microgram of yeast [BJ1551] alkaline carbonate-treated membrane preparation was incubated with varying amounts of the peptide KWDPA(farnesyl)CV[4,5-^{3}H]IA for 20 min at 37°. The labeled C-terminal tripeptide cleavage product was separated from unprocessed peptide by elution from a C_{18} reversed-phase sample cartridge with 25% acetonitrile/0.1% TFA (see Direct Assay section). (Inset) Values of K_m and V_{max} were determined from a Lineweaver–Burke plot of the data.

Materials

Peptide: KWDPA(*S-trans,trans*-farnesyl)CV[4,5-^{3}H]IA (47 mCi/mmol), 0.1 m*M* in 5% acetonitrile
Reaction buffer: 100 m*M* Tris-HCl, pH 7.4, containing 1 m*M* PMSF and 4 m*M* *o*-phenanthroline, prepared fresh
Sample preparation cartridge: UNIBOND C_{18} (Analtech, Newark, DE; Cat. No. 01-10)
Vacuum manifold: Analtech (Cat. No. 02-50)
Tubes: borosilicate (10 × 75 mm)
Solvents: Water/0.1% trifluoroacetic acid (TFA), 25% acetonitrile/0.1% TFA, 80% acetonitrile/0.1% TFA

Method. In advance, dispense 0.5-nmol portions of labeled peptide with 20 μl of 1 mg/ml bovine serum albumin (BSA) in 1.5 ml microcentrifuge tubes. Reduce the peptide/BSA solution to dryness in a Speed-Vac concentrator and store at −80°.

Resuspend an aliquot of the labeled peptide in 40 μl reaction buffer with PMSF and *o*-phenanthroline. Dilute the alkaline carbonate-treated yeast membrane preparation to 0.2 mg/ml in SST buffer (see above under Source of Enzyme). Initiate the reaction by adding 10 μl diluted yeast membrane preparation (2 μg protein) and transfer to 37° for 20 min. Terminate the reaction by placing tubes in a 65° water bath for 5 min.

Prepare the C_{18} sample cartridges by placing them in a vacuum manifold and applying 3 ml of each of the following in order: 80% acetonitrile/0.1% TFA; 25% acetonitrile/0.1% TFA; and water/0.1% TFA. Apply the heat-inactivated reactions to the sample cartridges, followed by 0.2 ml water/0.1% TFA. Elute the tritium-labeled tripeptide with 2.5 ml of 25% acetonitrile/0.1% TFA into a 10 × 75 mm collecting tube. Determine the amount of radioactivity by liquid scintillation counting.

Considerations. A control incubation without added membrane extract should be included in the experiment. The amount of eluted radioactivity that is not membrane protein-dependent should be subtracted from the values obtained from reactions incubated with membrane preparation. Typically, the value obtained from the no-extract control is about 2% of the total membrane protein-dependent radioactivity eluted from the cartridge.

Yeast membrane preparations possess considerably more RACE activity than do amphibian or mammalian extracts (Table I). Therefore, in assays with these membrane preparations, we incubate at least 10 μg membrane protein with the labeled peptide for 40–60 min.

When assaying several samples, we utilize a vacuum manifold equipped with fittings for up to 14 sample cartridges. A 5-ml syringe can substitute when a small number of samples are processed. We have found that the sample cartridges can be reused several times. If the cartridges are reused, they should first be cleared of unreacted peptide by eluting with 5 ml of 80% acetonitrile/0.1% TFA. A typical assay with 8–12 samples requires about 2 hr for the reaction incubation and processing.

The peptide sequence of the radiolabeled substrate in our experiments was derived from the yeast a-factor pheromone encoded by the *MFA1* gene. On application onto the C_{18} resin, the unproteolyzed farnesylated peptide and both proteolytic products are retained. The C-terminal tripeptide radiolabeled product elutes between 10 and 20% acetonitrile. The farnesylated peptide with or without the C-terminal tripeptide elutes between 50 and 80% acetonitrile. These parameters have been very reliable for a-factor peptides. However, retention/elution conditions for other peptides or other preparative cartridges may differ.

High-Performance Liquid Chromatography-Based Assay

High-performance liquid chromatography (HPLC) has been utilized for both qualitative and quantitative analysis of CaaX peptide proteolysis. The technique can be employed to monitor generation of the amino (N)- or carboxyl (C)-terminal peptide proteolytic products.

The reaction products should first be separated from the reaction mix to avoid application of crude membrane lipids and proteins onto the HPLC column. Recovery of the prenylated proteolyzed products from the reaction mix may be accomplished by organic extraction with butanol[9] or chloroform.[10] To monitor release of the C-terminal tripeptide proteolytic product, a radiolabeled peptide substrate is necessary because the tripeptide cannot readily be separated from the heterogeneous components present in the membrane preparation. We describe two methods to follow either N- or C-terminal endoproteolytic products of the reaction.

Identification of Amino-Terminal Reaction Product(s)

Materials

HPLC system: We use a Hewlett-Packard 1050 quaternary pump and variable wavelength detector
Solvent A: Water/0.1% TFA
Solvent B: Acetonitrile/0.083% TFA
Column: Vydac C_{18} reversed-phase, 4.6 × 250 mm, 10 μ (218TP104)
Protease inhibitor stock (50×): 50 m*M* PMSF, 200 m*M* 1,10-phenanthroline in methanol
Peptide substrate: KGVFWDPA(*S-trans,trans*-farnesyl)CVIA 1 m*M* in 25% acetonitrile
Water-saturated butanol

Method. Dispense 4 μl (4 nmol) portions of the prenylated peptide substrate stock into 1.5-ml microcentrifuge tubes. Add 80 μl of 100 m*M* Tris-HCl, pH 7.4, and 2 μl of PMSF/*o*-phenanthroline stock solutions to each tube. Initiate the reaction by adding 50 μg of yeast membrane protein in 20 μl.

Incubate the reaction at 37° for 40 min. Terminate the reaction by adding 150 μl of water-saturated butanol. Vortex the tubes for 15 sec and let them stand for 15 min at room temperature. Vortex the tubes again for 15 sec and centrifuge in a microcentrifuge for 3 min. Recover the butanol (top) layer and transfer to a new tube. Repeat butanol extraction of the original reaction mix with another 150 μl of water-saturated butanol. Combine both butanol extracts and evaporate to dryness in a Speed-Vac concentrator. Store at 4° until HPLC analysis is performed.

At the time of analysis, resuspend the samples in 25% acetonitrile/0.1% TFA and apply them to the reversed-phase column. Elute the peptides from the column with a linear 35–65% acetonitrile (solvent B) gradient with a 1 ml/min flow rate over 20 min. Peptide elution is monitored by absorbance at 214 nm.

Considerations. Because of the nonpolar nature of aaX terminal sequences, proteolytic removal of the tripeptide generally results in an increase in polarity (decrease in retention time) of the prenylated peptide. For example, removal of the tripeptide VIA from KGVFWDPA(*S-trans,trans*-farnesyl)CVIA decreases the retention time from 16.0 to 15.1 min (Fig. 5).

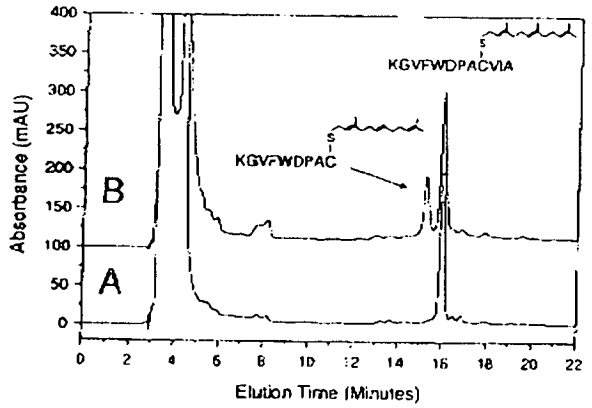

FIG. 5. Reversed-phase HPLC analysis of the amino-terminal endoproteolytic products of a farnesylated peptide. Ten nanomoles of the a-factor lipopeptide KGVFWDPA(farnesyl)CVIA was incubated with 20 μg yeast alkaline carbonate-treated membrane preparation (AC-P100) for zero (A) or 4 hr (B) at 37°. Elution of the peptides was monitored by absorbance at 214 nm. The identities of the peaks are shown and were confirmed by mass spectrometry.

Obviously, this generalization will not apply to all peptides, and the elution gradient may need to be adjusted. The identity of the reaction products should be verified by HPLC purification and mass spectrometry. Synthesis of the appropriate peptide standards also greatly assists in determining elution conditions for the particular peptide under investigation.

Additional protease activities in the cell extract are potential complications. Carboxypeptidase Y, a vacuolar serine protease, is present in yeast S100 fractions and will remove the terminal three residues from farnesylated peptides *in vitro*.[4,5] This activity can be eliminated by a *prc1* mutation, by a *pep4* mutation, or with a serine protease inhibitor such as PMSF.[13] A metalloendopeptidase activity present in yeast S100 fractions that is active on prenylated peptides has also been characterized.[6] The metalloendopeptidase is effectively inhibited by the metal chelator *o*-phenanthroline. Thus, the proper combination of mutations and inhibitors in studies of yeast RACE eliminates nearly all contaminating activities.

Identification of Carboxyl-Terminal Reaction Product(s)

The HPLC method can be employed to monitor release of the C-terminal tripeptide from a prenylated peptide substrate (Fig. 6). This procedure was also utilized to distinguish between an endo- and exoproteolytic mechanism of the membrane-bound protease.[4] Because the released tripeptide is susceptible to secondary proteolysis,[8] inclusion of additional protease inhibitors in the reaction buffer is warranted.

Materials. The following are needed in addition to materials listed above.

Peptides: KWDPA(*S-trans,trans*-farnesyl)CV[4,5-^{3}H]IA, VIA, IA
Reaction buffer: 100 m*M* Tris-HCl, pH 7.4, containing the inhibitors PMSF (1 m*M*), *o*-phenanthroline (4 m*M*), EDTA (1 m*M*), leupeptin (10 μ*M*), pepstatin (1 μ*M*), aprotinin (10 μ*M*), and E64 (10 μ*M*; see Ref. 21)
Sample preparation cartridge: SPICE C_{18} (Analtech)
Centricon-30 filter (Amicon, Danvers, MA)
Solvents: Water/0.1% TFA, 25% acetonitrile/0.1% TFA, 80% acetonitrile/0.1% TFA
3.0-ml syringe

Method. Carry out a protease assay utilizing a prenylated peptide substrate radiolabeled at one of the C-terminal tripeptide residues, as described in the preceding section. For the protease assay, use the reaction buffer listed here containing additional protease inhibitors. Also include in the reaction the synthetic tripeptide VIA (0.1 mg/ml); which will act both as a carrier and as a competitive inhibitor to prevent secondary proteolysis.

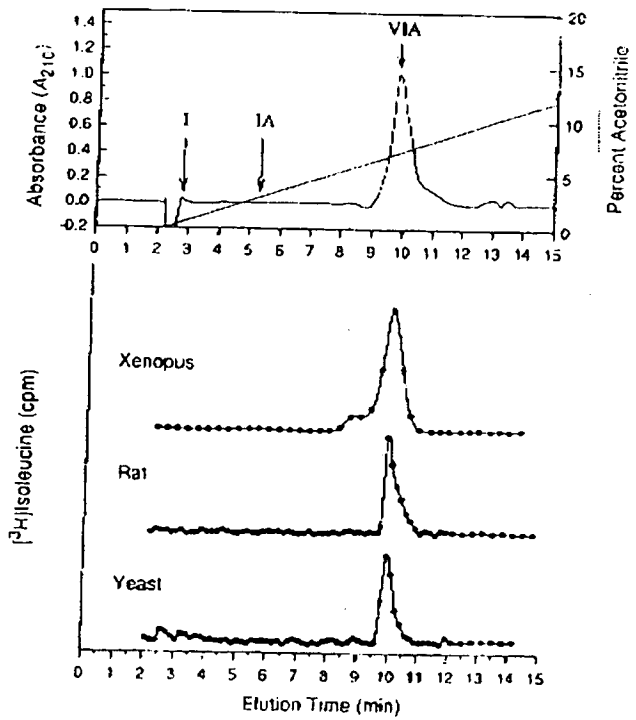

FIG. 6. Reversed-phase HPLC analysis of the carboxyl-terminal endoproteolytic products of a farnesylated peptide. Yeast and rat crude membrane preparations were incubated with the peptide KWDPA(farnesyl)CV[4,5-^{3}H]IA which was coupled to CH-Sepharose [M. N. Ashby, D. S. King, and J. Rine, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4613 (1992)]. *Xenopus* oocyte membrane preparation (45 μg) was incubated with 1 nmol KWDPA(farnesyl)CV[4,5-^{3}H]IA for 2 hr at 37° (see section on HPLC methods). The *Xenopus* experiment produced around 7000 counts/min (cpm) of cleaved product, which was applied to the reversed-phase column. Fractions were collected at 20-sec intervals. Detection of the standards was monitored by absorbance at 210 nm. Elution times of Ile (I) and Ile-Ala (IA) were determined in a separate parallel experiment and are also shown.

Scale the reaction such that at least 2000 disintegrations/min (dpm) of tritiated product is formed. Terminate the reaction by heating to 65° for 5 min.

Prepare a C_{18} cartridge by passing 3 ml of each of the following in order through the cartridge: 80% acetonitrile/0.1% TFA, 25% acetonitrile/0.1% TFA, and water/0.1% TFA. Apply the reaction mixture to the sample cartridge and begin collecting the effluent. Connect a 3-ml syringe to the cartridge and pass 1.5 ml of 25% acetonitrile/0.1% TFA through the cartridge into a collecting tube. Place the sample in a vacuum chamber to remove acetonitrile.

To remove the high molecular weight components from the reaction mix prior to HPLC analysis, centrifuge the aqueous sample in a Centricon-30 filtration unit at 5000 rpm (Sorvall SS34 rotor) for 20 min. Add 0.1 ml water to the top part of the filter and repeat the centrifugation. Collect the material passing through the filter and evaporate to dryness in a Speed-Vac concentrator.

Resuspend the sample in 100 μl of 10% acetonitrile/0.1% TFA. Determine the radioactivity content in a 5-μl portion to verify that enough labeled product is present. Apply the balance (95 μl) onto the HPLC column. We elute the sample from the reversed-phase column with a linear 0–40% acetonitrile (solvent B) gradient over 45 min with a flow rate of 1 ml/min. At the appearance of the solvent front, begin collecting fractions in vials at 10- or 20-sec intervals. Add 4 ml of liquid scintillation fluid to each vial and determine content of radioactivity by liquid scintillation counting.

To identify the C-terminal proteolytic products, chromatograph synthetic peptide standards in parallel and determine the respective elution times. Under these conditions the tripeptide VIA elutes at 10.0 min and the dipeptide IA at 5.2 min, and free isoleucine (I) is not retained on the column and elutes with the solvent front at 2.6 min (Fig. 6).

Considerations. As mentioned in the preceding section, the specific peptide elution conditions in the C_{18} sample preparation cartridge were determined for an a-factor peptide and may need adjusting for other peptides. On the other hand, the a-factor peptide was an adequate substrate for the endoprotease from all species tested.

Determination of CaaX Proteolysis *in Vivo*

The first structural determinations of prenylated proteins were performed on the mating pheromones from *Rhodosporidium*[22] and *Tremella*.[23,24] More recently, the detailed structure of the mature processed a-factor from *Saccharomyces* was reported which identified that farnesylation, proteolysis, and carboxylmethylation had taken place.[25] Heilmeyer

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and co-workers[26] elucidated the primary structure of phosphorylase kinase by mass spectrometry of the purified protein. Interestingly, they found that the mature α and β subunits of the enzyme are prenylated but not further proteolyzed or methylated.

Characterization of the posttranslational processing events *in vivo* usually has involved metabolic labeling experiments. Because C-terminal proteolysis of a CaaX sequence is required to provide a substrate for the methyltransferase, methylation indicates that proteolysis has occurred. To measure methylation, yeast cells are grown in the presence of *S*-adenosyl [*methyl*-^{3}H]methionine (SAM). In mammalian cells [*methyl*-^{3}H]methionine must be substituted for SAM as SAM is taken up very poorly,[27] and methionine is converted to SAM *in vivo*. The proteins of interest are immunopurified and directly analyzed by SDS–gel electrophoresis or are further proteolytically degraded. The resulting amino acids or peptides can be fractionated by HPLC or SDS–gel electrophoresis. Methyl esters are quantitated by a vapor-diffusion assay.[27] This approach has been used to detect posttranslational processing of yeast Ras2p[19,28,29] and mammalian p21^{ras},[30–32] p21^{rho} proteins,[33] p22^{rac},[34] the γ subunit of transducin,[35] and lamin B.[36,37]

Analysis of *in vivo* labeled proteins by SDS–gel electrophoresis often detects multiple protein species representing intermediates in posttranslational processing.[19,31,32] To identify proteolytic intermediates, Gutierrez and co-workers[31] engineered a unique tryptophan codon into the last coding position of p21^{ras}. By labeling COS cells harboring the construct with either [^{35}S]methionine or [^{3}H]tryptophan, they were able to identify directly intermediates that had undergone C-terminal proteolysis.

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Summary

We have described several quantitative and qualitative assays that have been utilized to learn the basic properties of RACE and amphibian and mammalian counterparts. Owing to powerful genetic tractability, high specific activity, and an apparently well-conserved substrate specificity, yeast is an attractive organism in which to study RACE. Efforts are currently in progress to characterize the functional role of the endoproteolytic processing step of many essential proteins.

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[21] Yeast STE14 Methyltransferase, Expressed as TrpE–STE14 Fusion Protein in *Escherichia coli*, for *in Vitro* Carboxylmethylation of Prenylated Polypeptides

By CHRISTINE A. HRYCYNA, STEPHANIE J. WAIT, PETER S. BACKLUND, JR., and SUSAN MICHAELIS

Introduction

Prenylated proteins comprise a recently discovered class of posttranslationally modified proteins in eukaryotes, many members of which are initially synthesized in precursor form containing a C-terminal CaaX motif (where C is cysteine, a is usually aliphatic, and X can be one of several amino acids). The CaaX motif acts as a signal for a series of three ordered modifications including prenylation of the cysteine residue via a thioether linkage by either a farnesyl or geranylgeranyl moiety (depending on the identity of X), proteolytic removal of the three C-terminal aaX residues,

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Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity

(CAAX motif/carboxyl methylation/farnesylation)

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ABSTRACT **We have introduced a variety of amino acid substitutions into carboxyl-terminal CA_1A_2X sequence (C = cysteine; A = aliphatic; X = any amino acid) of the oncogenic [Val^{12}]Ki-Ras4B protein to identify the amino acids that permit Ras processing (isoprenylation, proteolysis, and carboxyl methylation), membrane association, and transformation in cultured mammalian cells. While all substitutions were tolerated at the A_1 position, substitutions at A_2 and X reduced transforming activity. The A_2 residue was important for both isoprenylation and AAX proteolysis, whereas the X residue dictated the extent and specificity of isoprenoid modification only. Differences were observed between Ras processing in living cells and farnesylation efficiency in a cell-free system. Finally, one farnesylated mutant did not undergo either proteolysis or carboxyl methylation but still displayed efficient membrane association (≈50%) and transforming activity, indicating that farnesylation alone can support Ras transforming activity. Since both farnesylation and carboxyl methylation are critical for yeast a-factor biological activity, the three CAAX-signaled modifications may have different contributions to the function of different CAAX-containing proteins.**

An association with the plasma membrane is critical for Ras transforming activity (1–3), and this association is promoted by a series of three closely linked posttranslational modification steps signaled by the consensus carboxyl-terminal CA_1A_2X motif (C = cysteine; A = any aliphatic amino acid; X = any amino acid) present in all Ras proteins. Addition of the 15-carbon farnesyl isoprenoid to the cysteine of the CAAX sequence is followed by proteolytic removal of the AAX residues and carboxyl methylation of the now terminal cysteine residue. Mutant Ras proteins lacking either the cysteine or the AAX residues are completely blocked in processing and are cytosolic and completely nontransforming. Posttranslational processing is critical for Ras function, but the precise contribution of each of the three CAAX-signaled processing steps to Ras membrane association and transforming activity remains to be established.

The enzymes responsible for Ras processing are now beginning to be characterized. A cytosolic Ras farnesyltransferase activity, identified in both mammalian (4–6) and yeast (7, 8) cells, requires recognition of only the CAAX sequence to farnesylate the cysteine residue. In contrast to farnesyltransferase, the enzymatic activities for the AAX proteolysis (9) and carboxyl methylation (9–12) steps have been detected in the membrane component of fractionated cells and tissues.

In vitro studies with both synthetic peptides and chimeric Ras proteins have provided details of the sequence requirements for Ras farnesyltransferase modification. The residue at the A_1 position can vary, while a much more restricted set of A_2 and X residues permits efficient isoprenoid modification (13–15). The X residue also specifies whether the protein is modified by a farnesyl or by a geranylgeranyl group (14, 16–20).

While *in vitro* prenylation studies have been important for defining the sequence requirements for a functional CAAX motif, characterization of all three CAAX-signaled processing steps under physiological conditions with full-length proteins remains to be done. Furthermore, *in vivo* analyses will be important to establish the relationship between each CAAX-signaled processing step and Ras transforming activity. Therefore, we have used mouse NIH 3T3 cells as an "*in vivo*" system to address the following questions. (*i*) Does the Ras farnesyltransferase show *in vivo* the same specificity for CAAX residues that it does *in vitro*? (*ii*) What are the sequence requirements for efficient AAX proteolysis and carboxyl methylation? (*iii*) What are the relative contributions of the three different CAAX-signaled modifications to Ras membrane association and transforming activity? Our results identify differences between farnesylation efficiency *in vitro* and Ras processing *in vivo*, demonstrate a different sequence requirement for farnesylation versus AAX proteolysis, and suggest that farnesyl addition alone is sufficient for Ras membrane association and transforming activity.

MATERIALS AND METHODS

Generation of Ki-Ras4B CAAX Mutant Constructs. For these studies, we chose the Ki-Ras4B protein, which does not undergo the palmitate modification present in the three other human Ras proteins (21, 22), to avoid any complications due to differences in palmitoylation efficiency that may occur from mutations in the CAAX sequence. Polymerase III (Stratagene) or *Taq* polymerase chain reaction (PCR) DNA amplification using mutagenic oligonucleotides of an oncogenic [Val^{12}]Ki-Ras4B cDNA sequence was done to generate mutant sequences encoding single amino acid substitutions (see Table 1) in the carboxyl-terminal CVIM sequence (residues 185–188). As characterized previously (22), this Ki-Ras cDNA sequence encodes an additional N-terminal 10 residues from vector-derived sequences, and the resulting chimeric Ki-Ras4B protein migrates at ≈24 kDa in an SDS/polyacrylamide gel. This distinct mobility allowed us to clearly distinguish both processing and membrane association of the chimeric Ki-Ras4B proteins from that of the endogenous Ras proteins. All mutated Ki-Ras4B sequences were completely verified by dideoxy sequencing (23) and

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Abbreviations: CAAX, protein sequence where C = cysteine, A = aliphatic, and X = any amino acid; MVA, mevalonate; SAM, *S*-adenosyl-L-methionine.

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introduced into the pZIP-NeoSV(x)1 retrovirus vector (24) to generate pZIP-rasK constructs of each CAAX mutant.

Cell Culture and Transfection Analysis. NIH 3T3 cells were transfected by the calcium phosphate technique (22). To determine focus-forming activity, cells were transfected with 10, 100, or 1000 ng of pZIP-rasK plasmid DNA per 60-mm dish, and transformed foci were quantitated after 14–20 days. For protein analysis, transfected NIH 3T3 cells were selected and established in growth medium containing G418 at 400 μg/ml.

Subcellular Localization and Analysis of Posttranslational Processing. To determine the degree of membrane association and processing of the different CAAX mutants, G418-selected NIH 3T3 cells expressing each mutant [Val^{12}]Ki-Ras4B protein were labeled overnight with [^{35}S]methionine/cysteine (Tran^{35}S-label, ICN) or [5-^{3}H]mevalonolactone (≈25 Ci/mmol, NEN) at 200 μCi/ml (1 Ci = 37 GBq). [5-^{3}H]Mevalonolactone is converted to [^{3}H]mevalonate (MVA) *in vivo*. Metabolic labeling was done in the presence or absence of 50–100 μM compactin to inhibit biosynthesis of MVA, precursor of isoprenoid groups (25). Fractionation analysis was done as described (22). The cytosolic (S100) and membrane-containing (P100) fractions were immunoprecipitated with anti-Ras rat monoclonal antibody Y13-259 (26), then resolved by SDS/PAGE (18-cm-long gels), followed by fluorography. Carboxyl methylation of [Val^{12}]Ki-Ras4B proteins was analyzed on Y13-259-immunoprecipitated proteins isolated from cells labeled metabolically with L-[*methyl*-^{3}H]methionine (80 Ci/mmol; NEN) at 200 μCi/ml (27–29). After SDS/PAGE and fluorography, the labeled Ki-Ras4B bands were excised and incubated with 1 M NaOH for 24 hr at 37°C and the ratio of volatile to stable ^{3}H cpm was quantitated.

***In Vitro* Processing Analysis.** *In vitro* translation and processing were done (9) with a pGEM-3Z (Promega) Ki-Ras4B construct and T7 RNA polymerase. Nuclease-treated reticulocyte lysate (Promega) contains Ras farnesyltransferase activity as well as MVA and farnesyl pyrophosphate for isoprenylation of exogenously added Ras proteins (9). Translation reactions were supplemented with 5 mM nonradioactive MVA (for complete farnesylation) and with microsomal membranes (Promega) for efficient AAX proteolysis and carboxyl methylation when appropriate. Translations were done in the presence of [^{35}S]methionine/cysteine (1 mCi/ml) and methionine-free amino acid mix (Promega) to detect Ki-Ras4B expression. Translations were done with [^{3}H]MVA (1 mCi/ml) or *S*-adenosyl-L-methionine (SAM, 1.6 mCi/ml) (82 Ci/mmol; NEN) to detect isoprenylation or carboxyl methylation, respectively. Additionally, when indicated, 50 μM compactin or 2 mM *S*-adenosyl-L-homocysteine (Sigma) was included to prevent farnesylation (25) or carboxyl methylation (12), respectively.

To characterize the individual modification steps, translation and processing were done in 50-μl reaction volumes with the following four conditions: (*i*) unprocessed—reticulocyte lysate alone in the presence of 50 μM compactin [under these conditions, significant Ras isoprenylation (band 2) will still occur due to the presence of sufficient endogenous amounts of MVA or farnesyl pyrophosphate]; (*ii*) farnesylation only—reticulocyte lysate alone supplemented with 5 mM nonradioactive MVA; (*iii*) farnesylation with proteolytic removal of AAX residues—reticulocyte lysate with MVA and microsomal membranes with *S*-adenosylhomocysteine; and (*iv*) full processing—reticulocyte lysate with MVA and microsomal membranes. After translation and radiolabeling, 10 μl of the [^{35}S]methionine/cysteine-labeled samples or 25 μl of the [^{3}H]MVA- or [^{3}H]SAM-labeled samples was added to high-SDS/Tris RIPA buffer (0.05 M Tris, pH 7.0/0.15 M NaCl/0.5% SDS/1% sodium deoxycholate/0.01% Nonidet P-40/1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride) (total reaction volume 250 μl), immunoprecipitated with Y13-259 antibody, and analyzed by SDS/PAGE (35-mm-long gels) followed by fluorography.

RESULTS

Nature of the A_2 and X Residues Important for Ras Transforming Activity. To determine the requirements for a functional Ras CA_1A_2X sequence, single amino acid substitutions were introduced into [Val^{12}]Ki-Ras4B residue 186 (A_1), 187 (A_2), or 188 (X) to generate 22 different mutant proteins (Table 1). When the constructs encoding the mutants were transfected into NIH 3T3 cells, a range of transforming activities, from fully transforming to completely nontransforming, was observed in focus-formation assays (Table 1). Potent transforming activity was retained with all of the substitutions at A_1. In contrast, acidic or basic amino acid substitutions at either A_2 or X caused drastic reductions in, or loss of, transforming activity. In general, a good correlation was observed between the relative amount of Ki-Ras4B protein present in the crude membrane (versus cytosol) fraction and the transforming activities of the different mutant proteins (Table 1).

Differential Ki-Ras4B Processing Results in Three Distinct Electrophoretic Species. We next wanted to determine which

Table 1. Transforming activity, membrane association, and processing of Ki-Ras4B CAAX mutants

Substitution				Transforming activity*	Membrane association†	Processing‡
C	A_1	A_2	X			
C	V	I	M	1.00	++++	3
S	V	I	M	0.00	−	1
C				0.00	−	1
C	D	I	M	1.09	++++	3
C	C	I	M	1.01	++++	3
C	G	I	M	1.11	+++	1, 3§
C	Y	I	M	0.80	++++	3
C	V	D	M	0.00	−	1
C	V	C	M	1.10	++++	3
C	V	G	M	0.08	+	1, 2
C	V	Y	M	0.55	++	1, 2
C	V	K	M	0.00	−	1
C	V	I	D	0.32	++	1, 3
C	V	I	C	0.85	++++	3
C	V	I	G	0.89	++++	3§
C	V	I	Y	0.68	++++	3§
C	V	I	K	0.00	−	1
C	V	I	R	0.00	−	1
C	V	I	E	0.04	++	1, 3
C	V	I	S	1.27	++++	3
C	V	I	N	1.19	++++	3
C	V	I	T	1.04	++++	3
C	V	I	Q	0.79	++++	3
C	V	I	L	0.96	++++	3
C	V	I	F	0.82	++++	3

*Relative focus-forming activity in NIH 3T3 cells. Values were normalized to the focus-forming activity of [Val^{12}]Ki-Ras4B (4.2 × 10^{3} foci per μg of DNA) and represent the average of three to five independent assays.

†Quantitation of the fraction of Ki-Ras4B protein present in the particulate (P) versus soluble (S) fraction after fractionation and SDS/PAGE analysis: ++++, >90%; +++, 60–90%; ++, 30–60%; +, 5–30%; −, <5% (based on visual determination of at least two independent fractionation experiments).

‡Electrophoretic species of Ki-Ras4B protein detected on SDS/PAGE: Band 1, unprocessed; band 2, partially processed (isoprenylated only); band 3, complete (isoprenylated and carboxyl methylated) (see Fig. 1).

§Complete at 16 hr, but incomplete at 15 min [wild-type (CVIM) protein is completely processed after 15 min].

specific processing steps were critical for Ki-Ras4B membrane association and transforming activity. Our initial analyses of the expression of Ki-Ras4B CAAX mutant proteins in NIH 3T3 cells identified a variety of electrophoretic profiles on SDS/PAGE, with each CAAX mutant expressing various amounts of three species of distinct electrophoretic mobility. To determine whether these three species corresponded to differentially processed forms of Ki-Ras4B, we analyzed the posttranslational modifications corresponding to each electrophoretic species by using an *in vitro* reticulocyte lysate system supplemented with microsomal membranes (9).

Using the four conditions described in *Materials and Methods*, we were able to isolate each posttranslational processing step *in vitro* (Fig. 1). The three electrophoretic species of Ki-Ras4B are the unprocessed protein (band 1); the partially processed forms, which are farnesylated only, or farnesylated and proteolyzed (band 2); and the completely processed, farnesylated, proteolyzed, and carboxyl-methylated protein (band 3). Thus, the modifications of farnesylation and carboxyl methylation, but not proteolysis, account for the altered electrophoretic mobility of the fully processed Ki-Ras4B. The three species detected *in vitro* corresponded to the three electrophoretic species expressed in NIH 3T3 cells (data not shown). Although band 2 can represent either the farnesylated or the farnesylated and proteolyzed form of Ki-Ras4B *in vitro*, since proteolytic removal of the AAX residues of all mutants generates the same carboxyl-terminal farnesylcysteine substrate for carboxyl methylation *in vivo*, the band 2 species detected *in vivo* is believed to represent only the farnesylated form.

Differential Amino Acid Tolerance of the A_1, A_2, and X Positions for Processing *in Vivo*. To compare the ability of the A_1, A_2, and X residues to signal processing, the same amino acid substitutions were introduced into each position and processing was characterized in transfected NIH 3T3 cells. Like the wild-type protein, mutant proteins with cysteine substitutions at each position underwent complete processing, with no band 1 or 2 detected (Table 1). However, the introduction of aspartic or tyrosine residues had different

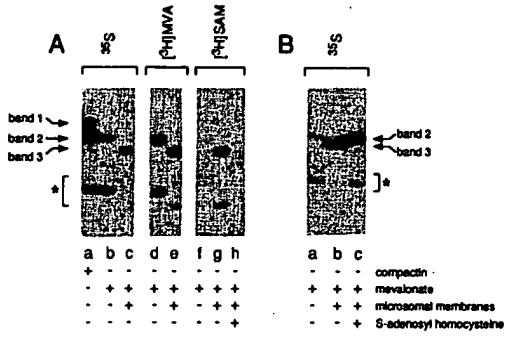

FIG. 1. Isoprenylation and carboxyl methylation account for the altered electrophoretic mobility of processed Ki-Ras4B protein. Translation and processing of Ki-Ras4B protein were done in rabbit reticulocyte lysates under the indicated conditions (see *Materials and Methods*). Radiolabeled samples were then immunoprecipitated with Y13-259 anti-Ras antibody and analyzed by SDS/PAGE using 35-cm-long gels to increase the resolution of the three electrophoretic forms of Ki-Ras4B: band 1, unprocessed protein; band 2, farnesylated protein, or farnesylated and proteolyzed protein; band 3, fully processed, farnesylated, proteolytically cleaved, and carboxyl-methylated protein. The read-through products of proteins initiated from the internal (authentic) ATG start site of the Ki-Ras4B coding sequence are designated by the bracket and star. The internally initiated protein undergoes the same modifications as the chimeric Ki-Ras4B; thus the amino-terminal leader sequence present on these mutants does not alter processing. Samples in *A* and *B* are from two separate experiments.

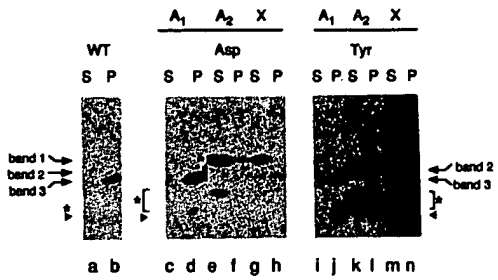

FIG. 2. Substitutions at A_2 and X, but not A_1, perturb processing and membrane association. NIH 3T3 cells expressing wild-type (WT) or mutant protein were metabolically labeled with [^{35}S]methionine/cysteine (200 μCi/ml) for 18 hr. The cell lysate was then separated into crude soluble (S) and particulate (P) fractions. Labeled proteins were immunoprecipitated from each fraction with the Y13-259 anti-Ras monoclonal antibody, resolved by SDS/PAGE, and detected by fluorography. Arrows designate the three electrophoretic forms of Ki-Ras4B as described in Fig. 1. Additional bands detected in the 21-kDa region represent either Ki-Ras4B protein expressed from the authentic ATG start site of Ki-Ras4B in the chimeric constructs (★) or endogenous Ras proteins (▸).

consequences for the three AAX positions. While neither substitution perturbed processing when made at the A_1 position (186D and 186Y) (Fig. 2, lanes c, d, i, and j), both impaired processing significantly when made at A_2 (187D and 187Y) (lanes e, f, k, and l). The 187D protein expressed *in vivo* was localized exclusively in the cytosol, and its mobility corresponded solely to that of the unprocessed protein (band 1) (lanes e and f). In contrast, the same substitution at X (188D) resulted in two electrophoretic species (lanes g and h). The major species was the unprocessed, cytosolic protein (band 1), while the second species corresponded to the fully processed, membrane-associated protein (band 3). A tyrosine substitution resulted in a partially processed protein when made at the A_2 position (band 2), but a fully processed protein (band 3) when introduced at X (lanes i–n). Both aspartic acid (187D) and lysine (187K) substitutions at A_2, as well as lysine (188K) and arginine (188R) substitutions at X, completely abolished processing (band 1), whereas the 188E protein was only partially processed (bands 1 and 3; data not shown). These data demonstrate that the amino acid requirements for a functional CAAX motif are not stringent for the A_1 residue, are most stringent for the A_2 residue, and are also restrictive for the X position.

The A_2 Residue Influences Both Farnesylation and Proteolysis, Whereas the X Residue Influences the Rate and Specificity of Isoprenylation Only. While certain amino acid substitutions at both the A_2 and X positions prevented complete posttranslational processing, the nature of the defect in processing was distinctly different at each position. For example, the mutant protein with a glycine at A_2 (187G) was expressed predominantly as unprocessed protein (band 1), with a minor amount of farnesylated protein (band 2); no fully processed protein was detected (Fig. 3*A*, lanes c and d). Similarly, the A_2 tyrosine mutant (187Y) was efficiently farnesylated (band 2), but was not further processed to the mature form (band 3) (lanes e and f). The band 2 forms of both 187G and 187Y proteins were radiolabeled with [^{3}H]MVA, and ≈50% of the farnesylated species was present in the membrane fraction (Fig. 3*B*, lanes c–f). Further analysis of both mutant proteins (band 2) showed an absence of methylation, as measured by the ratio of volatile to stable counts derived from [*methyl*-^{3}H]methionine, when compared with the fully processed Ki-Ras4B protein (data not shown). Thus, farnesylation alone was sufficient to promote significant Ki-Ras4B membrane association.

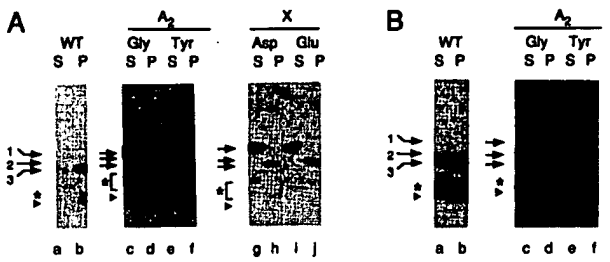

FIG. 3. Mutations at A_2 perturb both farnesylation and proteolytic removal of AAX, whereas mutations at X perturb only farnesylation. Cells expressing wild-type (WT) or mutant Ki-Ras4B proteins were labeled metabolically with either [^{35}S]methionine/cysteine (*A*) or [^{3}H]MVA (*B*), separated into soluble (S) and particulate (P) fractions, and resolved by SDS/PAGE. Labeled proteins were detected by fluorography. Arrows designate the three electrophoretic forms of Ki-Ras4B as described in Fig. 1. The internally initiated (★) or endogenous (▶) Ras proteins are designated as described in Fig. 2.

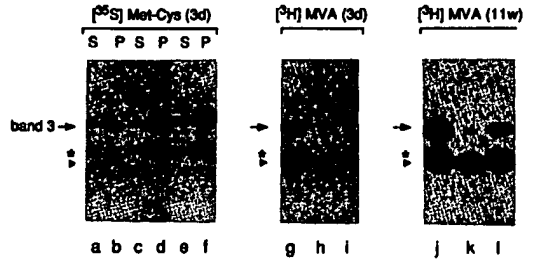

FIG. 4. Differential metabolic labeling with [^{3}H]MVA of X-position mutant proteins. Cells expressing wild-type Ki-Ras4B (lanes a, b, g, and j) or the 188L (lanes c, d, h, and k) or 188G (lanes e, f, i, and l) mutant were labeled metabolically with either [^{35}S]methionine/cysteine (lanes a–f) or [^{3}H]MVA (lanes g–l) and processed as described in Fig. 2. All lanes represent 3-day autoradiographic exposures, except lanes j, k, and l, which were 11-week exposures. Arrow indicates the location of the fully processed Ki-Ras4B. Internally initiated (★) or endogenous (▶) Ras proteins are designated as in Fig. 2. Note that the internally initiated forms of the 188L and 188G mutants are also poorly labeled with [^{3}H]MVA.

In contrast to the partially processed band 2 profiles of the A_2 mutant proteins, partially processed X-position mutants (188D and 188E) displayed different profiles consisting of two forms (Fig. 3*A*, lanes g–j), one of which corresponded to the unprocessed, cytosolic protein (band 1), but the other of which corresponded to the fully processed, membrane-associated protein (band 3). Thus, these substitutions perturbed only the rate or degree of farnesylation, but the farnesylated form was then completely processed to the mature form. Finally, pulse–chase analysis (data not shown) showed that some X substitutions (e.g., 188G and 188Y) perturbed the rate but not the steady-state degree of processing (Table 1). Therefore, the sequence requirements for farnesylation and proteolysis are clearly different, with the X residue influencing primarily the farnesylation step whereas the A_2 residue is important for both efficient farnesylation and AAX proteolysis.

Under the metabolic labeling conditions used in these studies, while proteins expected to be modified by a farnesyl isoprenoid were labeled efficiently by [^{3}H]MVA, proteins expected to be modified by a geranylgeranyl group were very poorly labeled (20, 29). The poor labeling of geranylgeranyl-modified proteins is believed to reflect not the degree of isoprenoid modification but rather the significantly lower specific activity of the geranylgeranyl pyrophosphate versus farnesyl pyrophosphate pools generated *in vivo* during our 16–18 hr labeling. A comparison of the degree of radiolabeling of the farnesylated wild-type Ki-Ras4B protein and the geranylgeranyl-modified 188L protein provides a striking example of this differential radiolabeling. Metabolic labeling with [^{35}S]methionine/cysteine indicated that the two proteins were expressed at comparable levels and were converted predominantly to the fully processed form (Fig. 4, lanes a–d). However, parallel cultures labeled with [^{3}H]MVA showed incorporation into the wild-type protein that was readily detectable after a 3-day autoradiographic exposure, whereas no [^{3}H]MVA incorporation was detected in the 188L mutant (lanes g and h). While the endogenous Ras proteins were efficiently labeled with [^{3}H]MVA, a faint band of the 188L Ki-Ras4B mutant was detected only after an 11-week autoradiographic exposure (compare lanes j and k). We have observed that, like leucine, the substitution of glycine (lanes i and l), threonine, phenylalanine, tyrosine, and asparagine (data not shown) at the X position also resulted in fully processed, membrane-associated proteins that displayed very weak metabolic labeling by [^{3}H]MVA. By contrast, mutant proteins with the X substitutions of cysteine, serine, and glutamine all underwent complete processing and exhibited efficient metabolic labeling with [^{3}H]MVA (data not shown). Thus, it is likely that proteins with the latter substitutions retain modification by farnesylation, while proteins with the former substitutions are altered in their isoprenoid specificity and are modified instead by a geranylgeranyl isoprenoid.

DISCUSSION

Our data on the processing *in vivo* of 22 Ki-Ras4B CAAX mutants are generally consistent with farnesylation results *in vitro* but also identify several important differences. As *in vitro*, we observed that the A_1 position was more tolerant than the A_2 position for a variety of amino acid substitutions. Basic and aromatic residues were tolerated at A_1 but not at A_2. Substitutions at A_1 and A_2 were found to influence prenylation efficiency but not specificity. Substitutions at X influenced the efficiency and specificity (farnesylation versus geranylgeranylation) of isoprenoid addition. Overall, our observations *in vivo*, together with results from studies *in vitro*, suggest that the A_1 and A_2 positions do not require aliphatic amino acids. Therefore, the CAAX motif should more appropriately be referred to as a CXXX motif.

One significant difference between farnesylation efficiency *in vitro* and isoprenylation and transforming activity *in vivo* was observed with aromatic substitutions at the A_2 position. A recent study (15) determined that CAAX tetrapeptide sequences with aromatic amino acids at the A_2 position were potent inhibitors of, but not substrates for, Ras farnesyltransferase activity *in vitro*. Such peptides represent good candidates for inhibitors of Ras farnesyltransferase because they would not be modified and inactivated by farnesylation. However, we found that a Ki-Ras4B CAAX mutant protein terminating in CVYM (187Y) did undergo efficient isoprenylation (>95%) when expressed in NIH 3T3 cells. These results suggest the possibility that an additional Ras farnesyltransferase(s) other than the presently identified activity may exist. Alternatively, they may reflect the possibility that the activities of Ras farnesyltransferase may not be identical *in vitro* and *in vivo* or may differ with tetrapeptides versus intact full-length proteins.

We observed that all mutant Ki-Ras4B proteins with X residues that should allow efficient farnesyl modification as predicted *in vivo* (i.e., where X = S, C, Q, or M) exhibited potent transforming activities. However, the effect on transforming activity of a few X-position substitutions did not reflect farnesylation efficiency *in vitro*. For example, CAAX peptides terminating in either leucine or phenylalanine were

neither effective inhibitors of nor substrates for Ras farnesylation *in vitro* (13–15, 17) but, instead, were efficiently geranylgeranylated (14, 16, 18–20). We observed that Ki-Ras4B mutant proteins in which X = L or F retained full transforming activity (0.82–0.96 relative focus-forming activity). One explanation for the apparent discrepancy between the *in vitro* farnesylation results and the transforming activity of these mutants is that, although they are not farnesylated, they are geranylgeranylated *in vivo*, and Ras transforming activity can be facilitated by either isoprenoid. Consistent with this possibility, Ras transforming activity can be promoted equally by either farnesylation or geranylgeranylation (20, 30). Thus, CAAX sequences with X-position residues that are able to signal efficient isoprenoid modification, independent of whether a farnesyl or geranylgeranyl moiety is added, would be expected to exhibit strong transforming activity.

Substitution of tyrosine at A_2 (187Y) resulted in a significant impairment of the AAX proteolysis and carboxyl methylation steps, without abolishing farnesylation. The efficient membrane association (≈50%) and transforming activity (0.55 relative focus-forming activity) of the 187Y mutant protein suggests that isoprenoid modification alone is sufficient for oncogenic Ras function. These results are consistent with the observation that carboxyl methylation, although important, is not essential for the membrane association or function of yeast RAS2 (31). Thus, pharmacologic inhibitors of farnesylation, but not of proteolysis or carboxyl methylation, are likely to be effective for blocking oncogenic Ras function in tumors. These results contrast with the observations that both farnesylation and carboxyl methylation are critical for the biological activity of yeast a-type mating factor (32–34). Thus, the importance of isoprenylation, proteolysis, and carboxyl methylation for protein function is likely to be different for each protein that undergoes CAAX-signaled modifications.

While both the A_2 and X positions showed similar intolerance to amino acid substitutions with respect to effect on transforming activity, the consequences of A_2 and X residue substitutions for processing were distinctly different. Substitutions at X perturbed isoprenylation but not AAX proteolysis. In contrast, substitutions at A_2 perturbed both isoprenylation and AAX proteolysis. Thus, the sequence requirements to signal these two modifications are clearly different.

Recent studies *in vitro* have begun to establish the sequence requirements for a functional CAAX motif for isoprenoid modification. The *in vivo* studies described here provide further characterization of the sequence requirements for the CAAX motif to signal isoprenylation and AAX proteolysis. The differences that we have observed between Ras processing in living cells and farnesylation in a cell-free system demonstrate the importance of evaluating the activity of prenyltransferases under physiological conditions. The generation of partially processed Ras proteins has allowed us to begin to distinguish the role of each modification in facilitating Ras membrane association and transforming activity. Further studies will be required to determine the precise contribution of each CAAX-signaled processing step to the biological function of Ras proteins and to determine whether these contributions are the same for isoprenylated proteins such as additional members of the Ras superfamily, the nuclear lamins, and the γ subunits of heterotrimeric guanine nucleotide-binding proteins (3).

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